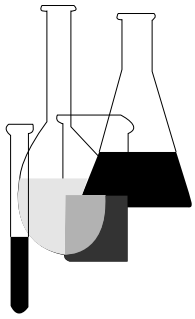
United States
Environmental Protection
Agency

Prevention, Pesticides
and Toxic Substances
(7101)

EPA 712–C–96–161
April 1996

Ecological Effects Test Guidelines

OPPTS 850.5100
Soil Microbial Community Toxicity Test



“Public Draft”

INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

Public Draft Access Information: This draft guideline is part of a series of related harmonized guidelines that need to be considered as a unit. *For copies:* These guidelines are available electronically from the EPA Public Access Gopher (gopher.epa.gov) under the heading ‘‘Environmental Test Methods and Guidelines’’ or in paper by contacting the OPP Public Docket at (703) 305–5805 or by e-mail: guidelines@epamail.epa.gov.

To Submit Comments: Interested persons are invited to submit comments. By mail: Public Docket and Freedom of Information Section, Office of Pesticide Programs, Field Operations Division (7506C), Environmental Protection Agency, 401 M St. SW., Washington, DC 20460. In person: bring to: Rm. 1132, Crystal Mall #2, 1921 Jefferson Davis Highway, Arlington, VA. Comments may also be submitted electronically by sending electronic mail (e-mail) to: guidelines@epamail.epa.gov.

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OPPTS 850.5100 Soil microbial community toxicity test.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) **Background.** The source material used in developing this harmonized OPPTS test guideline is 40 CFR 797.3700 Soil Microbial Community Toxicity Test (proposed in the FEDERAL REGISTER of September 28, 1987 (52 FR 36350)).

(b) **Purpose**. This guideline is intended for use in developing data on the toxicity of chemical substances and mixtures (''test substances'') subject to environmental effects test regulations. The guideline prescribes a test using natural soil samples to develop data on the toxicity of test substances to microbial populations indigenous to the soil. The EPA will use data from these tests in assessing the hazard of a test substance to the environment.

(c) **Definitions.** The definitions in section 3 of TSCA and 40 CFR Part 792—Good Laboratory Practice Standards apply to this guideline. The following definitions also apply:

Ammonification is conversion of organic nitrogen compounds to ammonia (NH_3) or to ammonium ion (NH_4) compounds, performed by a variety of microorganisms in soil and water.

Carbon dioxide (CO_2) efflux is the evolution of CO_2 gas from substrates mineralized by microbial action—indicative of respiration.

EC X is the experimentally-derived test substance concentration that is calculated to affect X percent of the test species.

kiloPascal (kPa) is a unit of pressure in the meter-kilogram-second system equivalent to one newton per square meter (i.e., 1 Pa $\times$ 1,000) used as a measure of water availability in soils.

Mineralization is the complete or ultimate degradation by microorganisms of organic material to form inorganic end-products, e.g., carbon dioxide, water, chloride, ammonium, nitrates, or orthophosphate.

Nitrification is the oxidation of ammonium salts to nitrites (NO_2) and nitrates (NO_3), performed by relatively specialized microorganisms.

Surface soil is that layer of soil representing the top 15 cm of the area to be sampled, excluding the litter horizon.

(d) **Test procedures**—(1) **Summary of the test**. Surface soil is sieved and supplemented with ground, dry alfalfa. The test substance, if soluble, is added as a solution to moisten the soil, or is added in a manner

that best simulates its anticipated mode of entry in nature. All soil samples are then incubated in darkness at approximately 22 °C. On days 5 and 28 after introduction of the test substance, samples are analyzed for NH_3 and NO_3 content to establish ammonification and nitrification values, respectively, and for CO_2 efflux as an indication of microbial respiration.

(2) **Application of test substance**. (i) Deionized or glass-distilled water should be used in making stock solutions of a water-soluble test substance. Sufficient quantities of each concentration should be made as needed to minimize storage time and disposal volume. A constant volume of the stock solution should be added at the beginning of the test to each soil sample designed to receive the test substance.

(ii) A test substance that is insoluble in water, but which can be suspended in an aqueous solution by a carrier, should be added, with the carrier, to those soil samples designated to receive the test substance. The carrier should be soluble in water, nontoxic to microbial life at the concentration applied, and used in the minimum amount required to dissolve or suspend the test substance. There are preferred carriers; however, acetone, gum arabic, ethanol, and others have been used extensively in testing herbicides, plant growth regulators, fungicides, and other chemical substances that affect plants. Any such carrier may be used for this test, providing it neither enhances nor inhibits the activities of the soil microbes. Carrier controls should be included in the experimental design and tested simultaneously with the test substance.

(iii) A water-insoluble test substance for which no nontoxic, water-soluble carrier is available should be dissolved in an appropriate volatile solvent. The solution should be mixed with the ground alfalfa soil supplement, then placed in a rotary vacuum apparatus and evaporated, leaving a uniform coating of the test substance on the alfalfa. A portion of the alfalfa should be weighed and the test substance should be extracted with the same organic solvent. Then the test substance should be assayed before the alfalfa is mixed with the soil in the test containers. Solvent controls (i.e., alfalfa treated only with solvent) should be included in the experimental design and tested simultaneously with the test substance.

(iv) If the test substance is not readily soluble in water or in another commonly-used carrier, and is known to be applied or transported in nature directly to the soil as a previously prepared liquid or powder, it should be mixed, in its liquid or dry form, directly into the soil samples. Mixing must be thorough, however, to ensure equal distribution of the test substance throughout each test sample.

(3) **Range-finding test.** (i) A range-finding test should be conducted to establish (A) if definitive testing is necessary and (B) the concentrations of test substance to be used in the definitive test.

(ii) If the maximum concentration of test substance to which the soil microbial community is likely to be exposed in nature can be predicted, soil samples should be treated with concentrations that are 0.1, 1, and 10 times the anticipated environmental exposure concentration. On days 5 and 28 after introduction of the test substance, the effects of treatment should be assessed as the CO_2 efflux rate and the NO_3 and NH_3 concentrations per gram of dry soil in treated samples, relative to untreated controls and, if applicable, carrier controls, and to values in freshly sieved (pretreatment) soil. Should reasonable predictions of anticipated environmental exposure concentrations not be possible, soil samples should be exposed to a series of widely-spaced concentrations (e.g., 1, 10, 100, 1,000, 10,000 μg/g) of the test substance. In general, the highest concentration in the series should not be less than 1,000 μg/g, although for water-soluble test substances, it is recommended that levels not exceed 50 percent of the saturation concentration. As before, CO_2 efflux and NO_3 and NH_3 concentrations should be compared with controls.

(iii) The test should consist of exposing at least two samples of soil from the same source to each concentration of test substance and to each control, with the exception of the controls for which one sample will suffice. To be appropriate for this guideline, a soil should possess a pH of 4 to 8, an organic matter content between 1 and 8 percent, a cation exchange capacity greater than 7 meq/100 g, and consist of less than 70 percent sand. Soils to which fertilizer or pesticide(s) have been applied within the past 24 months should be avoided. Soil collections should be restricted to the surface soil. Large objects should be removed manually, and the remaining soil allowed to air-dry until sievable (approximately 12 percent water content), after which it is passed through a 2-mm mesh screen. For each sample, an amount of soil equivalent to approximately 50 g oven-dry weight should be placed in an inert container. Widemouth jars (for example, glass canning, 0.5 pint or 110-mL capacity) are adequate for this purpose. At least one of these samples, considered to be the control, should be extracted immediately to determine NH_3 and NO_3 content (see paragraph (d)(4)(vii) of this guideline). Alfalfa, dried and ground to pass through a 0.6-mm mesh screen, should be added (0.3 g) to each remaining sample and the sample should be thoroughly mixed. Water content of the soil should be adjusted to approximately 10 kPa by adding distilled water containing the desired concentration of test substance (in carrier, if necessary). If insoluble in both water and commonly used carriers, the test substance should be mixed into the soil as a solid and the appropriate amount of water added subsequently. The test containers may be covered with 0.13 μm (0.5 mil) polyethylene to minimize water loss, yet permit gas exchange, or left open and watered to their original weight every 7 days. Regardless, the test substance should be applied only during the original watering.

(iv) Controls should receive an equal volume of water without the test substance. If a carrier solvent is required to dissolve or suspend the test substance, a carrier control (i.e., solvent in water without the test substance) should also be included. Should the test substance be a powder that is mixed directly into soil and subsequently moistened with distilled water, the control should receive an equal volume of such water only. All samples should be incubated in darkness at 22 °C (or at the temperature to which the microorganisms are most accustomed in their soil environment).

(v) Of the soil samples prepared for each concentration of test substance or control, one sample should be assayed after 5 days of exposure to the test substance for NH_3 and NO_3 content, and then discarded. A second sample should be analyzed the same day for CO_2 evolution and then reincubated (dark, 22 °C). On day 28, after exposure to the test substance, all remaining (reincubated) samples should be assayed first for CO_2 efflux and then again for nitrogen content.

(vi) The test substance should be chemically stable in distilled water or in any chemical substance used as a carrier.

(vii) No replicates are required, and nominal concentrations are acceptable unless definitive testing is not required.

(viii) Definitive testing is not necessary if the highest concentration of test substance tested (but not less than 1,000 μg/g) results in less than a 50 percent reduction of ammonification, nitrification, and CO_2 evolution; or if that concentration representing the analytical detection limit (if tested) results in greater than a 50 percent reduction of NH_3 and NO_3 content of the soil and of CO_2 generation.

(4) **Definitive test**. (i) The purpose of the definitive test is to determine whether the test substance is toxic to the community of microorganisms residing in a particular soil and, if so, to delineate its concentration-response curves and EC_{50s} for each of three variables (CO_2 evolution and NH_3 and NO_3 soil content) that indicate the capacity of the soil microbial community to decompose organic matter and release plant nutrients.

(ii) Preparations for the test should be made as described for the range-finding test (see paragraphs (d)(3)(iii) through (d)(3)(v) of this guideline) except that more samples of each soil source to be tested are required. As before, at least two series of soil samples should be prepared for each concentration; one series to be analyzed 5 days after exposure, the other to be analyzed on the 28th day after exposure. Series replicates of at least five concentrations of test substance, exclusive of controls, should be used for each of the two series. For each soil source tested, the concentration range should be selected to define, as closely as possible, the concentration-response curve between the EC_{10} and EC_{90} for each variable.

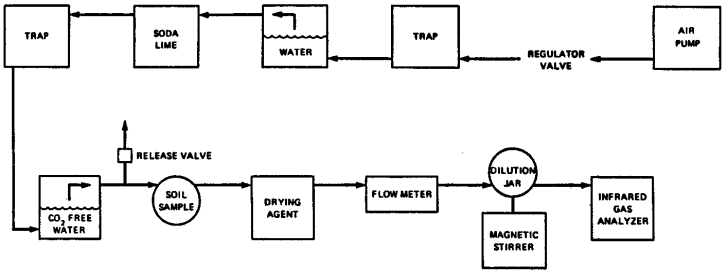
(iii) Every test should include controls consisting of the same distilled water, soil, and alfalfa supplement used in the treated soil samples, that none of the test substance is added. Environmental conditions should likewise be the same. If a carrier is needed to dissolve or suspend the test substance, a carrier control should also be included.

(iv) Test containers may be covered with polyethylene film (0.13-μm; 0.5-mil) to prevent water loss. Control containers should be handled identically to the test containers except that none of the test substance is added.

(v) The definitive test consists of testing soil (containing the natural microbial population) from a particular source with the test substance (see paragraph (d)(3)(iii) of this guideline). For a particular test substance, a test is the exposure of the selected soil to two identical series of five concentrations of the test substance in a minimum of five replicate containers per concentration, and includes appropriate controls, with analyses of CO_2, NH_3, and NO_3 on the 5th and 28th days after exposure to the test substance.

(vi) To measure CO_2 efflux, each container of test substance and control container is closed with a two-hole stopper fitted with Teflon tubes and twistcock connectors for attachment to the measurement apparatus. The apparatus (see Figure 1. following) should deliver a stream of humid, CO_2-free air to the test system, and the effluent air should be dried, diluted, and delivered for infrared gas analysis (IRGA). The period of incubation should be adjusted to match the CO_2 efflux rate with the detection capability of the IRGA, and may vary from 1 to 77 h. In lieu of IRGA, CO_2 may be trapped in a hydroxide solution and titrated, or measured by gas chromatography.

Figure 1.—FLOW DIAGRAM FOR THE IRGA METHOD OF DETERMINING CO_2 Evolution from Soil Samples



(vii) Accumulation of inorganic nitrogen is measured by extracting each soil sample with 80 mL of 1 N KCl. After adding KCl and shaking each container by hand to suspend the soil, sample containers should be placed on a rotary shaker at high speed for 1 h, then shaken again by hand to resuspend the soil. Samples should be filtered (Whatman 42 low-nitrate filter paper) and the extract (filtrate) should be analyzed for NO_3 and NH_3. Being rapid and precise, standard Autotechnicon analysis techniques are recommended. Acceptable alternative methods are available, however.

(viii) The assignment of soil containers to test substance concentrations should be random. In addition, placement of the containers in the incubation chamber should be randomized.

(ix) Temperature in the incubation chamber should be monitored continuously.

(5) **Analytical measurements**-(i) **Chemical**. For readily aqueous-soluble test substances, stock solutions of the test substance should be diluted with glass-distilled or deionized water to obtain the test solutions. Standard analytical methods should be used to establish concentrations of these solutions and should be validated before beginning the test. An analytical method is not acceptable if likely degradation products of the test substance, such as hydrolysis and oxidation products, give positive or negative interference. The pH of these solutions should also be measured before use.

(ii) **Numerical.** CO_2 efflux rates (micrograms per gram of dry soil/h) and NO_3 and NH_3 concentrations (micrograms per gram of dry soil) in treated samples should be determined and compared with values obtained from untreated controls, carrier controls (if a carrier is used), and from the freshly sieved, pretreatment soil. The significance of differences between means may be established using Duncan's Multiple Range Test. Means and standard deviations should be plotted for each treated sample

and each control. Appropriate statistical analyses should provide a goodness-of-fit determination for the concentration-response curves.

(e) **Test conditions**—(1) **Test species**. No particular species of test organisms are recommended for use in this test due to the emphasis placed on maintaining the natural state of the soil samples and their resident populations of microorganisms. The test organisms are, therefore, those that occur naturally in the soil, and no others are to be introduced.

(2) **Facilities**—(i) **Apparatus.** (A) Test chambers should provide adequate space and controls necessary to incubate numerous soil samples in total darkness at a constant temperature for prolonged periods of time. Chambers should be designed to prevent escape of internal air into the external environment other than through appropriate filtering material or media to prevent contamination of the external environment with the test substance.

(B) Laboratory facilities for test substance determinations should include nonporous floor covering; absorbent bench covering with nonporous backing, and adequate disposal facilities to accommodate test and wash solutions containing the test substance at the end of each test, and any bench covering, lab clothing, or other contaminated materials.

(ii) **Containers**. For each test, at least 60 to 70 soil containers (two series of five per concentration of test substance, two series of five for the control, and two series of five if a carrier control is necessary) should be used. In addition, soil to be extracted immediately as the control is most easily handled in an identical container. All containers used in each experiment should be of equal size and volume, possess the same configuration, and should be made of the same inert-material.

(iii) **Cleaning and sterilization.** (A) Soil containers and test solution storage containers should be cleaned before use. All equipment should be washed according to good standard laboratory procedures to remove any residues remaining from manufacturing or prior use. A dichromate solution should not be used for cleaning containers.

(B) If cleaning and rinsing of previously used soil containers has been thorough, the effects of any microorganisms remaining on the interior surface of the containers should be insignificant in the presence of the new test soil. Sterilization should not be necessary, but is considered an acceptable option.

(C) Soil treated with the test substance and solvent control soil should be discarded at the end of the experiment. Disposal should conform to applicable Federal regulations.

(3) **Test parameters**. Environmental conditions should be maintained as follows:

(i) Constant incubation temperature of 22 °C (or that temperature to which the microorganisms are most accustomed in nature).

(ii) Total darkness during incubation to prevent photosynthesis by algae or the growth of moss.

(f) **Reporting.** The final report should include, but not necessarily be limited to, the following information:

(1) Name and address of the facility performing the study and the dates on which the study was initiated and was completed, terminated, or discontinued.

(2) Objectives and procedures stated in the approved protocol, including any changes in the original protocol.

(3) Statistical methods used for analyzing the data.

(4) The test substance identified by name, Chemical Abstracts Service (CAS) registry number or code number, source, lot or batch number, strength, purity, and composition or other appropriate characteristics.

(5) Stability of the test and, if used, control substances under the conditions of administration.

(6) A description of the methods used, which should include the following:

(i) Description of environmental conditions, including type and size of incubation chamber and temperature used.

(ii) Description of test diluent/solvent if other than distilled water, e.g., if carrier is required.

(iii) Description of experimental design and/or arrangement of equipment, including a diagram, if complex.

(iv) Methods used to determine the placement of soil containers in the incubation chamber and the assignment of test concentrations to containers to ensure randomization of exposure.

(v) Frequency and methods of adding water to soil containers during the test period.

(7) A description of the test system used, including the source of the test soil, the type of ecosystem from which it was removed, its chemical and physical characteristics (mechanical analysis), and any available geological information including soil type (classification).

(8) A description of the amount of soil tested per concentration, number of replicates, carrier (if any), and incubation periods.

(9) The concentration of the test substance per unit dry weight of test soil when the test substance is dissolved in water, solubilized with a carrier, or coated on the alfalfa supplement and/or mixed into the soil.

(10) pH of the test solution applied to the soil samples. The reported results should include:

(i) The results of the range-finding test expressed as micrograms of CO_2 evolved per gram of dry soil per hour, and micrograms of each of NH_3 and NO_3 present per gram of dry soil, in treated and untreated samples. If the range-finding test indicated that the highest concentration of the test substance tested (but not less than 1,000 μg/g) had no effect on the test system, report the results by soil source and type and state that the test substance has a low potential for adversely affecting microbial functions in such soils. If the range-finding test indicated a greater than 50 percent reduction of the endpoints of the test at a concentration of the test substance that represents the analytical detection limit (if tested), report the results by soil source and type and state that the test substance is toxic to microbial life in such soils at concentrations at or below the analytical detection limit used in this study.

(ii) For the definitive test, the soil source and type, concentrations of test substance used (micrograms per gram dry soil), and data for the same variables used in the range-finding test (see paragraph (f)(1)(x)(A) of this guideline) should be reported.

(11) A description of all circumstances that may have affected the quality or integrity of the data.

(12) The name of the sponsor, study director, principal investigator, names of other scientists or professionals, and the names of all supervisory personnel involved in the study.

(13)A description of the transformations, calculations, or operations performed on the data, a summary and analysis of the data, and a statement of the conclusions drawn from the analysis. Results of the analysis of data should include the concentration-response curves with 95–percent confidence limits, the results of a goodness-of-fit test, e.g., X test, and EC50s.

(14) The signed and dated reports of each of the individual scientists or other professionals involved in the study including each person who, at the request or direction of the testing facility or sponsor, conducted an analysis or evaluation of data or specimens from the study after data generation was completed.

(15) The locations where all specimens, raw data, and the final report are stored.

(16) The statement prepared and signed by the quality assurance unit.